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Vascular Endothelial Growth Factor/Vascular Permeability Factor Is Temporally and Spatially Correlated with Ocular Angiogenesis in a Primate Model

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Ischemia often precedes neovascularization. In ocular neovascularization, such as occurs in diabetic retinopathy, a diffusible angiogenic factor has been postulated to be produced by ischemic retina and to lead to neovascularization of the retina, optic nerve, or iris. However, no angiogenic factor has been conclusively identified that satisfies this hypothesis. Vascular endothelial growth factor/vascular permeability factor, hereafter referred to as VEGF, is a likely candidate for an ocular angiogenic factor because it is a secreted mitogen, specific for endothelial cells, and is upregulated by hypoxia. We investigated the association of VEGF with the development of experimental iris neovascularization in the cynomolgus monkey. Following the production of retinal ischemia by laser occlusion of all branch retinal veins, VEGF was increased in the aqueous fluid, and the aqueous VEGF levels changed synchronously and proportionally with the severity of iris neovascularization. Northern analysis and in situ hybridization revealed that VEGF messenger RNA is upregulated in the ischemic retina. These observations support the hypothesis that ocular neovascularization is regulated by a diffusible factor and identify VEGF as a likely candidate for a retina-derived vascular permeability and angiogenesis factor in vivo. (Am J Pathol 1994, 145:574–584)

Angiogenesis, or the growth of new capillaries from existing blood vessels, occurs physiologically in ovulation and embryogenesis, and pathologically in ocular neovascularization (such as retinal vein occlusion and diabetic retinopathy), in rheumatoid arthritis, and in solid tumors. Insight into the mechanisms governing angiogenesis in one system are likely to apply to other angiogenic processes. Ocular neovascularization, at once visible and accessible, provides an ideal *in vivo* system in which to investigate the mechanisms that control angiogenesis.

Clinically, iris and retinal neovascularization are associated with areas of retinal capillary closure and ischemic retina, leading to the hypothesis that a factor diffusing from retinal ischemia is responsible for neovascularization.[1,2] Although angiogenic activity has been demonstrated from ocular tissues,[3,4] no specific angiogenic factor has been conclusively implicated in ocular angiogenic diseases. Vascular endothelial growth factor/vascular permeability factor (VEGF), a heparin-binding, dimeric protein, is an endothelial cell mitogen *in vitro*, and induces increased vascular permeability and angiogenesis *in vivo*.[5–9] Unlike basic fibroblast growth factor, another angiogenic factor found in the retina, VEGF is a secreted endothelial-selective mitogen.[8,10] In addition, VEGF has been demonstrated to be upregulated by hypoxia *in vitro*[11,12]

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and in solid tumors *in vivo*,[12–15] making it an appealing candidate for mediating ocular angiogenesis.

We have used a model of iris neovascularization in the nonhuman primate to investigate mechanisms that control angiogenesis. Laser photocoagulation was used to occlude the retinal veins, producing an ischemic retina similar to human clinical disease, and demonstrable by fundus fluorescein angiography. In a related model of retinal vein occlusion in the miniature pig, the affected retina has been demonstrated to be hypoxic using an O_2-sensitive microelectrode.[16] Normal primate iris has barely detectable vessels that do not leak fluorescein, although in inflamed eyes, mild leakage does occur. Four to seven days following laser vein occlusion, new iris vessels appear, characterized angiographically by rapid, diffuse fluorescein leakage from a vessel network.[17] Histological examination has confirmed the appearance of new iris vessels in this model, with thin-walled branching capillaries.[17] We have used this model to demonstrate a temporal and spatial association between VEGF and iris neovascularization.

Materials and Methods

Animal Procedures

All animals were cared for in accordance with the Association for Research in Vision and Ophthalmology Resolution on the use of animals for research, and guidelines established for animal care at the Massachusetts Eye and Ear Infirmary. Cynomolgus monkeys (*Maccaca fascicularis*) were anesthetized for all procedures with an intramuscular injection of a mixture of ketamine, 20 mg/kg (Ketalar, Parke-Davis, Morris Plains, NJ), diazepam, 1 mg/kg (Elkins-Sinn Inc., Cherry Hill, NJ), and atropine sulfate, 0.125 mg/kg (Gensia Laboratories Ltd, Irvine, CA). Supplemental anesthesia of ketamine (10 mg/kg intramuscular) assured a stable plane of general anesthesia. Proparacaine hydrochloride (0.5%) topical anesthetic drops (Alcon, Humacao, Puerto Rico) were administered before placement of any lid speculae and for pneumotonometry. Pupils were dilated as needed with 2.5% phenylephrine and 0.8% tropicamide drops. Animals were placed in a comfortable restraint device to allow head positioning for photography and angiography. Deeply anesthetized animals were killed with an intravenous injection of Somnlethal (JA Webster, Sterling, MA), a pentabarbital-based euthanasia solution approved by the American Veterinary Medical Association.

Primate Model of Iris Neovascularization

We used a cynomolgus monkey model of iris neovascularization adapted from a previously published model,[18] which simulates human retinal vein occlusion. Dye yellow (577 nm) laser light (Coherent Lambda Plus, Palo Alto, CA) was used to occlude all branch retinal veins in the eyes of cynomolgus monkeys. Fundus photography and fluorescein angiography, using 0.1 cc/kg of 10% sodium fluorescein via saphenous vein, was performed after laser vein occlusion. Angiography revealed venous occlusion retinopathy, with venous dilation, dot and blot hemorrhages, and areas of capillary nonperfusion, a hallmark of ischemic retina. Iris photography and fluorescein angiography were similarly performed using an adapter[19] mounted in front of a Canon CF-60ZA fundus camera (Lake Success, Long Island, NY). Angiograms were performed in a standard manner to obtain approximately 1 frame per second during the initial phase of the angiogram, and with both eyes photographed within the first 35 seconds after injection. Photographs were taken up to 2 minutes after injection, which captured the majority of the fluorescein leakage in this model.

A standardized grading system for iris neovascularization in this model has been developed, using standard photographs and angiograms (Miller et al. manuscript in preparation). This system analyzes the vessel density and degree of fluorescein leakage. Briefly, iris neovascularization is graded from 0 to 5, with grade 2 representing the threshold for classification as neovascularization. Grade 0 is a normal iris with few vessels visible and no leakage. Grade 1 has an increased vascular pattern without leakage and is typical of regressed neovascularization. A standard photograph 2A separates grades 2 and 3, with increasing vessel density and more rapid, early leakage of fluorescein. Mild, slow, leakage classed as grade 2 can be seen with inflammation, but higher grades are more specific for neovascular tissue. Grade 4 has enough density of neovascular tissue that the iris appears opaque within the first 35 seconds of the angiogram. Grade 5 has the characteristics of grade 4 plus ectropion uveae or glaucoma. The photographs and angiograms were coded by a technician, masked as to their date and animal, and then graded by two independent investigators. Using the grading system, the correlation between the two independent readers was 0.92.

Aqueous samples were obtained from sedated animals, using a 30-gauge needle attached to a siliconized 1-cc syringe passed through the limbus. After removal of aqueous fluid, a drop of gentamicin

sulfate was instilled into the fornix. Vitreous samples were obtained only after enucleation. Aqueous and vitreous samples were frozen at −4 C until they were assayed for VEGF.

Association of VEGF Level and Iris Neovascularization

A pilot study was performed to identify any relationship between VEGF level and iris neovascularization. Laser vein occlusion was performed in four eyes of two monkeys in the first experiment, which followed iris neovascularization grade (as described above) and aqueous VEGF levels for 35 to 37 days after laser. Fluorescein angiography was performed and aqueous samples were obtained every 6 days.

In a second set of experiments, a control was incorporated that simulated the laser injury without producing retinal ischemia. Four animals were used in which the right eye underwent laser vein occlusion and the left eye underwent "sham" laser, for a total of eight eyes. In the sham lasered eyes, laser spots were placed adjacent to the retinal veins without producing vein occlusion or retinal ischemia, using the same number of laser spots, with the same spot size, power, and duration. Thus each animal was its own control, with one ischemic eye, and one nonischemic eye. Fluorescein angiograms and VEGF sampling were performed every 2 to 4 days to characterize the changes as iris neovascularization developed. The animals were followed for 13 to 15 days after laser. One monkey suffered an anesthetic accident and died 5 days after laser, so that only six eyes were followed beyond 6 days.

VEGF Protein Assay

VEGF levels in the aqueous were measured using a two-site time-resolved immunofluorometric assay, originally developed to measure guinea pig VEGF,[20] and modified to measure human VEGF using homogeneous recombinant human VEGF as calibrators.[21] The antibodies were prepared against the N- and C-terminal peptides of VEGF.[9,22] It was assumed that there would be substantial cross-reactivity between the monkey and human VEGF. Subsequently, we have cloned and sequenced monkey VEGF and have found that although there are minor changes in the complementary (c)DNA sequence, the amino acid sequence is identical (Shima et al, manuscript in preparation). Antibody to the carboxy terminus of human VEGF was first immobilized on microtiter wells. Aliquots (50 μl) of samples to be assayed were incubated with gentle shaking overnight at 4 C with 50 μl of assay buffer in the wells. After washing the wells, 100 μl of assay buffer containing europium chelate-(Eu^{3+}) labeled antibody to the amino terminus of VEGF, was then added to the wells and incubated at room temperature for 2 hours. The wells were washed, an enhancement solution added, and fluorescence was measured in a time-resolved fluorometer. The Eu^{3+} chelate has a long decay time (>500 μs), which allows reading of fluorescence to be determined between 400 and 800 μsec in time-dependent fashion. Most endogenous nonspecific fluorescence occurs at shorter times, which improves the signal to noise ratio in this assay. The interassay coefficient of variation at 25 pM is 13.7%, and the minimal detection limit is 5.5 pM. Because both the biological samples and the calibrators are in an environment of assay buffer, there is little concern that VEGF detection in biological fluids such as aqueous would be poor. However, to confirm this, the VEGF immunoassay was performed on monkey aqueous to which a known quantity of VEGF was added, and the recovery was 94% (MW = 42 kd for VEGF calculations).

Statistical Methods

The VEGF level and iris grade were displayed as scatterplots. Each was plotted against time, and for the data from the second experiment, VEGF level was plotted against iris grade. Curves were drawn by connecting x-y medians, using cubic splines to approximate the x-y relationships.[23] Because each animal contributed both an ischemic and a nonischemic, sham measure of VEGF and iris grade at each time point, the measures were summarized as paired differences (ie, ischemic minus nonischemic value for each animal at each time point). This had the effect of "correcting" the vein-occluded eye for the "background" VEGF level or iris grade that is due to the laser injury. Because the control was the second eye of the same animal, other sources of variability were minimized. We looked to see if these corrected measures increased or decreased over time and to see if the corrected VEGF level and iris grade increased or decreased together, using analysis of variance[24] at successive time points contributed by each animal. Both VEGF level and iris grade were ranked before the analysis of variance because they were not normally distributed.[25]

Northern Analysis

Retinas were isolated from anesthetized animals before sacrifice and immediately frozen in liquid N_2 for

storage. The tissue was rapidly thawed by placing it into 5-ml, room temperature guanidinium buffer. The tissue was solubilized with a polytron and total RNA was isolated from retina using the guanidinium isothiocyanate/CsCl method.[26] RNA (15 μg per sample) was fractionated by electrophoresis on a 1.0% agarose-formaldehyde gel. The samples were transferred to nylon filters by capillary blotting overnight, and ultraviolet cross-linked. The filter was prehybridized for 4 hours at 42 C with 6× sodium chloride phosphate ethylenediaminetetraacetic acid, 5× Denhardt's, 50% formamide, 0.5% sodium dodecyl sulfate, and 100 μg/ml salmon sperm DNA followed by an 18-hour incubation with a random prime-labeled, 520-bp, *NcoI*/*BglII* fragment of the cloned human VEGF cDNA, a gift provided by Dr. Herbert Weich. Filters were washed once with 1× sodium chloride phosphate ethylenediaminetetraacetic acid, 0.1% sodium dodecyl sulfate (37 C), followed by two 10-minute washes with 0.1× sodium chloride phosphate ethylenediaminetetraacetic acid, 0.1% sodium dodecyl sulfate at 65 C. The same blot was stripped and reprobed with a 280-bp fragment of bovine 28S recombinant RNA cDNA labeled by random priming. The bands were visualized by autoradiography for 48 hours at −80 C.

In Situ *Hybridization*

The *in situ* hybridization protocol and the preparation of ^{35}S-labeled VEGF riboprobes and control sense riboprobes have been previously described.[14,27,28] Briefly, ^{35}S-labeled, single-stranded anti-sense and sense RNA probes for VEGF messenger (m)RNA were prepared. The anti-sense probe hybridizes with a region of VEGF mRNA coding sequence common to all four of the known splice variants of VEGF. Eyes for *in situ* hybridization were fixed for 4 hours in 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, at 4 C, and then transferred to 30% sucrose in phosphate-buffered saline, pH 7.4, overnight. Tissue was then embedded in paraffin, and 4-μ sections were hybridized overnight. The slides were washed with serial solutions before and after hybridization. Slides were then dehydrated, coated with emulsion, and stored. After a 2-week exposure, the emulsion was developed and the slides counterstained.

Results

Association of VEGF Levels with Severity of Iris Neovascularization

In a pilot experiment, four eyes of two monkeys were followed for 35 to 37 days after laser retinal vein occlusion. Fluorescein angiography was performed and aqueous samples were obtained every 6 days to measure the development of iris neovascularization and aqueous VEGF level. Figure 1, A and B, shows the relationship between aqueous VEGF levels and the grade of iris neovascularization in the four eyes, and time. There was substantial variability in the degree of iris neovascularization, and in the aqueous VEGF levels from monkey to monkey. However, it is apparent that both measures change over time, and there seems to be some temporal association between changing aqueous VEGF level and changing iris neovascularization. VEGF was undetectable in the aqueous before laser vein occlusion, rose above 30 pM as iris neovascularization developed, and then fell

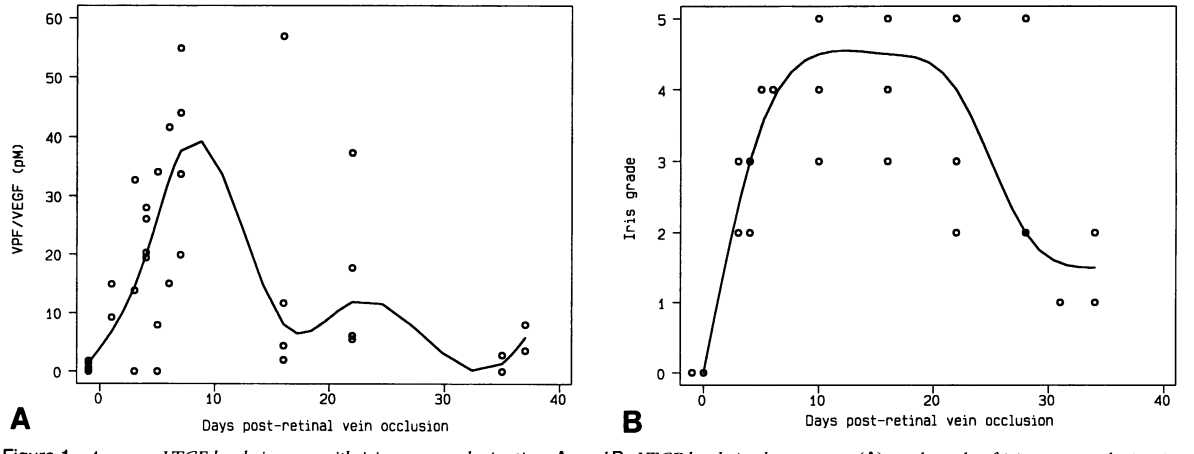

Figure 1. *Aqueous VEGF levels in eyes with iris neovascularization.* A *and* B: *VEGF levels in the aqueous* (A) *and grade of iris neovascularization* (B) *of four monkey eyes are compared over time after laser vein occlusion as a scatterplot with best fit curves. Although there is substantial variability between eyes, there seems to be an association between VEGF level and iris neovascularization. VEGF was undetectable in the aqueous before laser, the levels rise as neovascularization develops, and fall as the neovascularization regresses.*

to less than 10 pM as new vessels regressed. The level of VEGF detected in the aqueous as iris neovascularization developed was well above the concentration required for maximal stimulation of vascular endothelial cells *in vitro*. VEGF was undetectable in serum throughout the course of the experiment, making it unlikely that the VEGF measured in the aqueous was derived from the blood.

A second set of experiments was undertaken that incorporated a sham laser control to verify that increased aqueous VEGF and iris neovascularization was not due to a nonspecific response to retinal laser injury. In the sham eyes, or nonischemic eyes, laser spots were placed adjacent to the retinal veins without producing vein occlusion or retinal ischemia. The fundus appearance of an ischemic and a nonischemic eye is shown in Figure 2, A and D. Fluorescein angiography of an eye with laser vein occlusion demonstrated venous occlusion and ischemic retinopathy, with retinal capillary closure in the distribution of

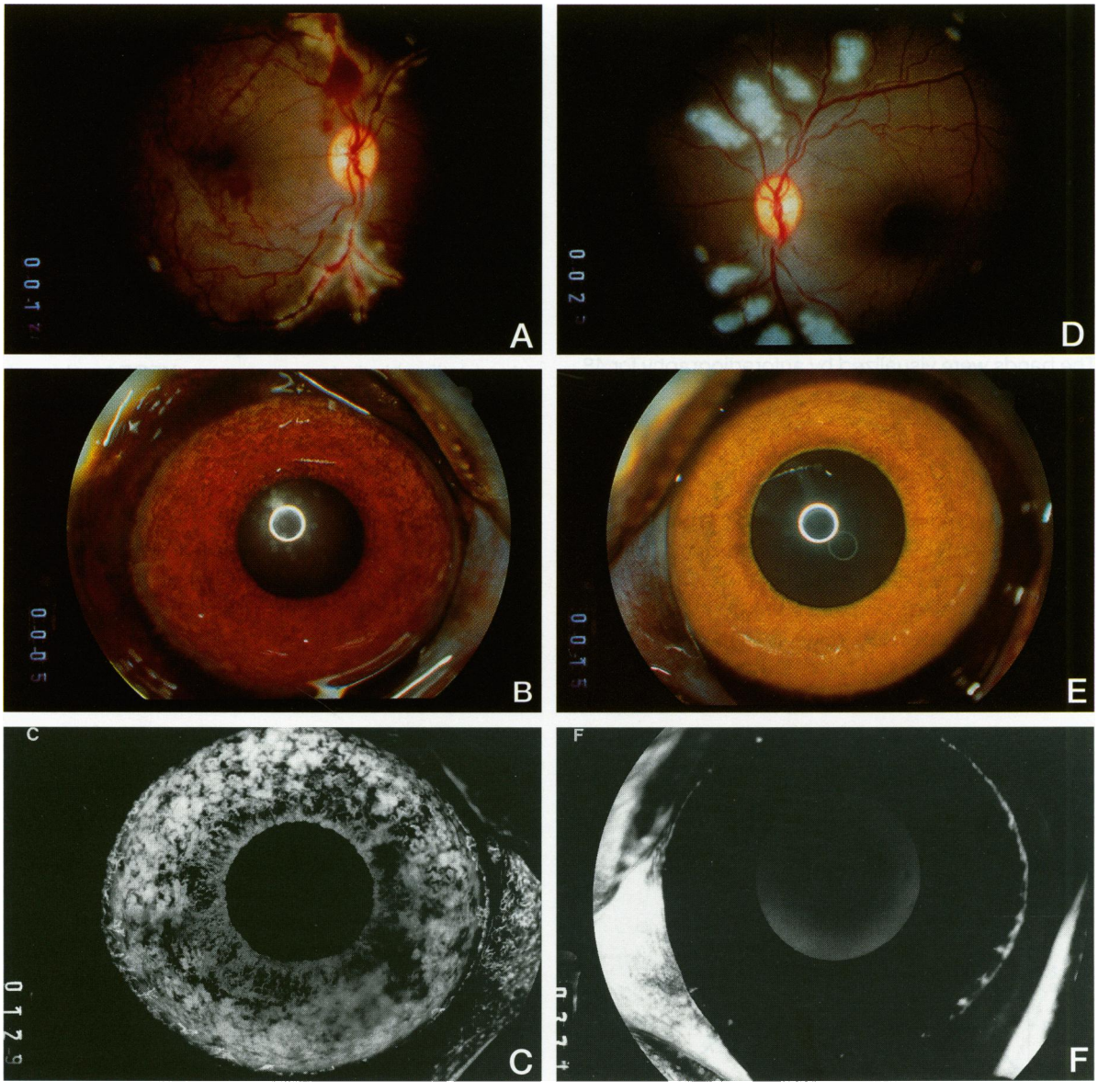

Figure 2. *Experimental iris neovascularization.* **A:** *Fundus photograph immediately after laser vein occlusion.* **B:** *New vessels are seen on the surface of the iris. Iris neovascularization appears 4 to 7 days after laser vein occlusion, increases, and persists for an average of 24 days, or leads to the development of neovascular glaucoma.* **C:** *Fluorescein angiography demonstrates new vessels on the surface of the iris, with a characteristic tortuous pattern, and abundant leakage of fluorescein into the anterior chamber (grade 3).* **D:** *Fundus photograph immediately after sham laser, in which laser spots were placed adjacent to the retinal vessels, to produce retinal injury but preserving normal retinal vasculature.* **E:** *The iris appears normal 12 days after sham laser.* **F:** *Fluorescein angiography of the iris in* **E** *demonstrates no leakage of fluorescein from the normal iris vessels (grade 0).*

the occluded veins, and fluorescein leakage and retinal edema in the posterior pole. Fluorescein angiography of a sham lasered eye demonstrated staining of the laser lesions with normal retinal vasculature. The severity of the laser injury is identical, and the different fundus appearance is due to disruption of the venous blood flow in the retina following laser vein occlusion. The eyes with laser vein occlusion and ischemic retina developed progressive iris neovascularization characterized by marked leakage of fluorescein from tortuous vessels (grade 3; Figure 2, B and C). Sham lasered eyes did not develop iris neovascularization (grade 0; Figure 2, E and F), but in some cases demonstrated mild fluorescein leakage (grade 2), probably secondary to mild inflammation induced by serial aqueous sampling.

Figure 3A shows how the VEGF levels in the ischemic (laser vein occlusion) and nonischemic (sham lasered) eyes change over time. VEGF was undetectable in the aqueous of eyes with sham laser and nonischemic retina, whereas it rose to 30 pM or more in the ischemic eyes. The corrected VEGF level (ischemic minus nonischemic) increased significantly over time ($P \leq 0.001$). Iris fluorescein angiograms were graded for degree of neovascularization in the ischemic and nonischemic eyes, and similarly plotted *versus* days after retinal laser treatment in Figure 3B. In this experiment, the eyes were only followed for 13 to 15 days after laser, and iris neovascularization was still increasing in the ischemic eyes. The sham lasered nonischemic eyes developed mild leakage on angiography (corresponding to a grade of 2), but did not progress, and the corrected iris grade increased significantly during this period ($P = 0.01$). Finally, the corrected VEGF level and iris grade over time are plotted against one another in Figure 3C. Whereas

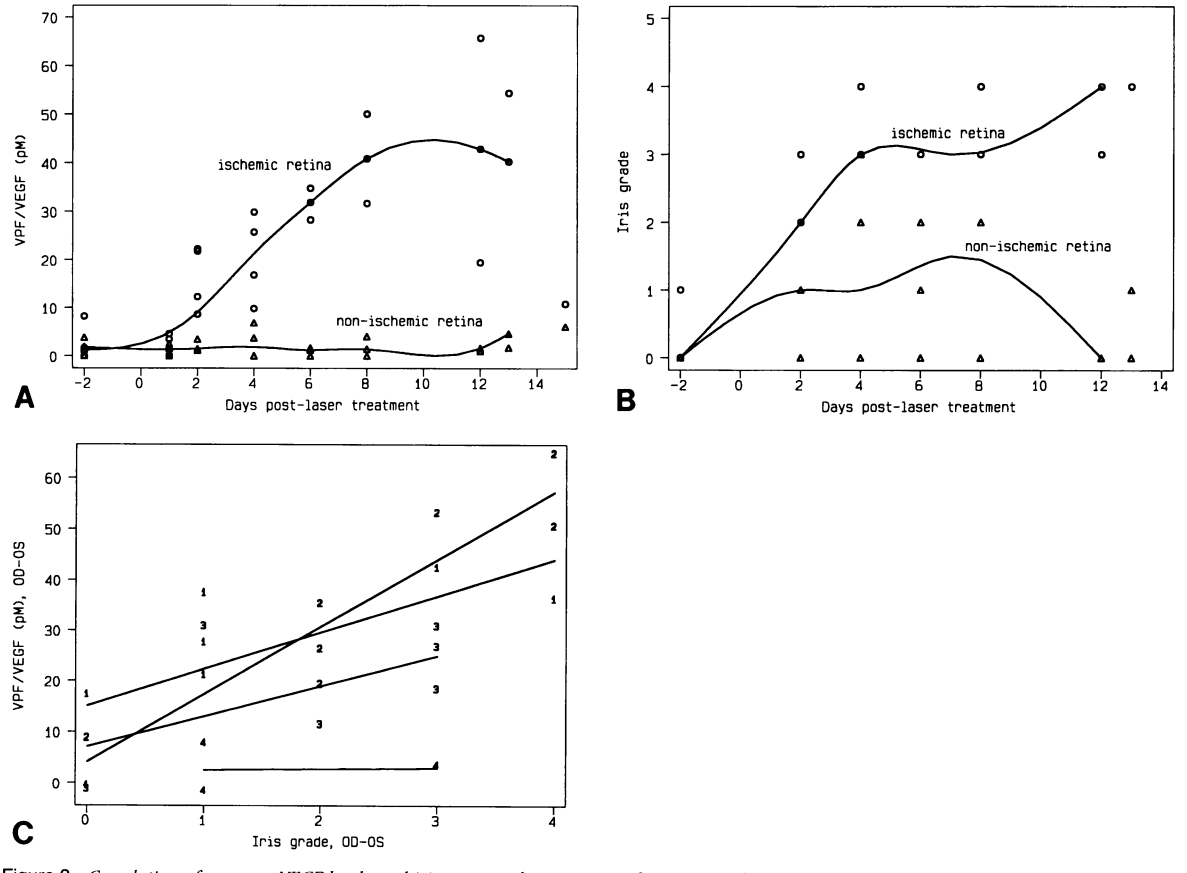

Figure 3. *Correlation of aqueous VEGF levels and iris neovascularization grade in eyes with laser vein occlusion* versus *sham laser.* **A:** *VEGF levels in the ischemic laser vein occlusion eyes (°), and nonischemic, sham lasered eyes (△) are compared over time as a scatterplot with best fit curves. The corrected VEGF level (ischemic minus nonischemic) increased significantly over time (P ≤ 0.0001). One monkey died on day 5 after laser, so that data after this time point is for six eyes only.* **B:** *Iris grade in the same group of eyes compared over time as a scatterplot with best fit curves (ischemic °; nonischemic △). Two of the sham lasered eyes developed grade 2 leakage, with mild, late leakage on angiography, probably secondary to mild inflammation induced by serial aqueous sampling. The corrected iris grade increased significantly over time (P = 0.013).* **C:** *The corrected VEGF level and iris grade over time are shown, with each number corresponding to a monkey. Whereas, there is substantial variation from animal to animal, there is a statistically significant relationship between increasing VEGF level and increasing neovascularization (P ≤ 0.0001). Monkey 4 died on day 5 after laser and was included in the analysis, although the curve from this animal clearly has a different slope. It can be postulated that there was insufficient time for much difference to develop between the two eyes (ischemic and nonischemic) in this animal.*

there is substantial variation from monkey to monkey, there is a statistically significant relationship ($P \leq 0.001$) between increasing VEGF level and increasing neovascularization. These results demonstrate that aqueous VEGF level and grade of iris neovascularization track together over time in this model of iris neovascularization.

Vitreous VEGF levels were obtained at the time of sacrifice from only a few animals throughout the experiments. In the five ischemic eyes assayed, the vitreous VEGF levels were equal to or higher than the levels measured in simultaneously obtained aqueous. This result supports the hypothesis that the VEGF is released by ischemic retina in the posterior segment of the eye, diffuses forward, and induces iris neovascularization. Vitreous levels in the nonischemic eyes were low or undetectable (data not shown).

Analysis of VEGF in Ischemic versus *Nonischemic Retinas*

To confirm that VEGF is produced by ischemic retina, the expression of VEGF mRNA was investigated by Northern analysis. Retinas were isolated from one eye with laser vein occlusion and iris neovascularization, and from one eye that received a sham laser and was without iris neovascularization. Two VEGF transcripts were identified in the ischemic retina, a distinct and high abundance band at 3.6 kb and a faint, low abundance band at approximately 3.9 kb. This pattern is in agreement with VEGF transcripts observed in other systems.[28,29] The authors have subsequently identified the monkey retina transcripts by polymerase chain reaction analysis to be the 121-kd and 165-kd forms of VEGF (Shima et al, manuscript in preparation). In contrast to the ischemic retina, VEGF mRNA was barely detectable in the nonischemic retina (Figure 4), consistent with upregulation of VEGF protein in ischemia.

Localization of VEGF mRNA in Ischemic Retina by in Situ *Hybridization*

In situ hybridization with a VEGF riboprobe was performed on ischemic and nonischemic retinas and demonstrated intense labeling of the inner nuclear layer of ischemic retina in eyes with iris neovascularization (Figure 5, A and B). In contrast, there was minimal labeling in the nonischemic retinas of eyes without iris neovascularization (Figure 5, C and D). No specific cellular labeling was seen with control sense probes and background levels were low (Figure 5, E and F). There was no definite labeling of iris tissue in either group of eyes. The retinal pigment epithelium bound both VEGF riboprobe and control sense probe, making interpretation in this cell layer difficult (data not shown).

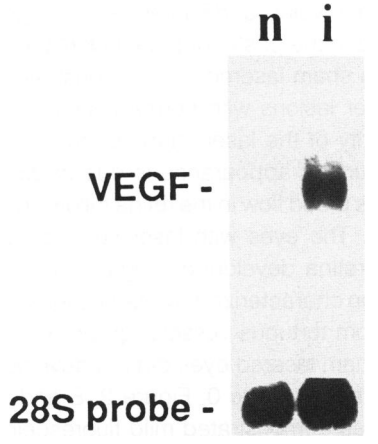

Figure 4. *Northern blot analysis of ischemic and nonischemic retinas. VEGF Northern blot of total cellular RNA of retinas isolated from eyes with ischemic retina and iris neovascularization* (lane i)*, and contralateral eyes with sham laser and nonischemic retina without iris neovascularization* (lane n) *probed for VEGF. Two VEGF transcripts were identified in the ischemic retina, a distinct and high-abundance band at 3.6 kb and a faint, low-abundance band migrating just above it at 3.9 kb.*

Discussion

Our findings demonstrate a clear temporal and spatial relationship between a secreted angiogenic factor, VEGF, and the development of heightened vascular permeability and ocular neovascularization *in vivo*. Before laser vein occlusion, the VEGF level in the aqueous was undetectable; within 2 days of laser treatment, the aqueous levels had risen, and neovascularization was detectable by 4 days. Aqueous levels of VEGF peaked in the second week after vein occlusion, coincident with the most severe neovascularization; the aqueous VEGF levels fell before regression of the neovascularization. When the aqueous VEGF levels were determined and angiography was performed every 2 to 4 days, the aqueous VEGF levels and the severity of iris neovascularization increased synchronously and proportionally. The increase in aqueous VEGF was not simply a response to laser injury inasmuch as control eyes receiving a sham laser treatment of equivalent extent did not have elevated VEGF. This is the first time, to our knowledge, that levels of an angiogenic factor have been shown to be related to the extent of neovascularization over time *in vivo*.

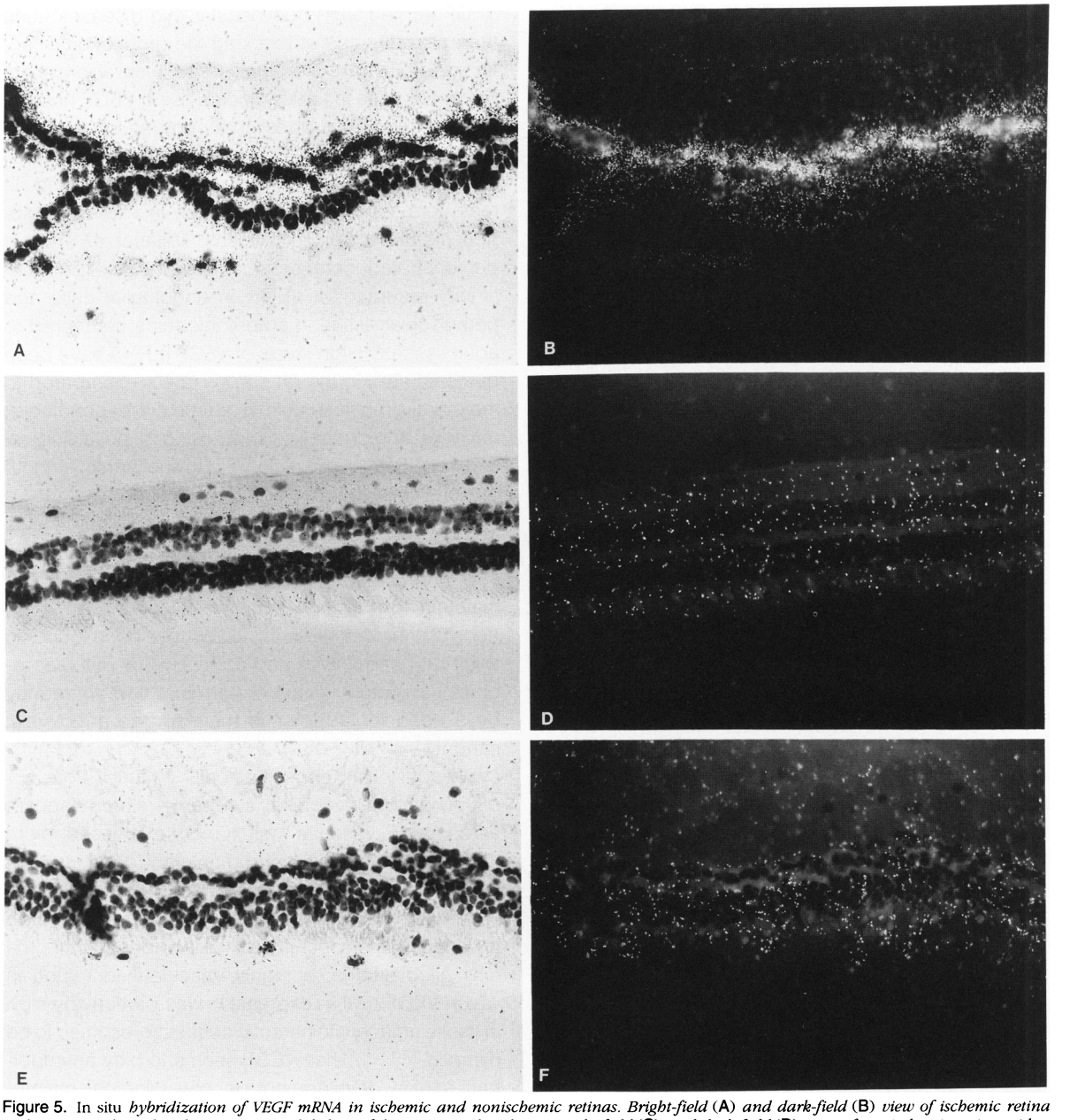

Figure 5. In situ *hybridization of VEGF mRNA in ischemic and nonischemic retinas. Bright-field* (**A**) *and dark-field* (**B**) *view of ischemic retina with a VEGF riboprobe, showing intense labeling of the inner nuclear layer. Bright-field* (**C**) *and dark-field* (**D**) *view of nonischemic retina with a VEGF riboprobe showing minimal labeling. Bright-field* (**E**) *and dark-field* (**F**) *of ischemic retina with a VEGF control sense riboprobe.*

The evidence for ischemic retina as the source of the increased aqueous VEGF is both indirect and direct. Serum VEGF remained undetectable through the course of the experiment, making it unlikely that VEGF was derived from the blood. Vitreous levels of VEGF were equal to or higher than aqueous levels in eyes with iris neovascularization, suggesting that the VEGF was being released from a source in the posterior segment. This is consistent with the hypothesis that VEGF is secreted from the retina into the vitreous, diffuses into the aqueous, where it induces iris neovascularization. More direct evidence implicating the retina as the source of the VEGF is provided by results of *in situ* hybridization and Northern analysis. Northern analysis of the total RNA isolated from ischemic and nonischemic retinas demonstrated a marked increased in VEGF mRNA transcripts only in the retina that was rendered ischemic by laser vein occlusion. *In situ* hybridization revealed that VEGF mRNA localized to the inner nuclear layer of the ischemic retina. Whereas the cell type expressing the VEGF mRNA could not be determined from these experiments, the

Mueller cell is a possible candidate, because its cell body is located in the inner nuclear layer, and it has cell processes that span the entire retina, filling spaces between axons and blood vessels in the nerve fiber layer. Equally likely are the mural cells (smooth muscle cells or pericytes) of the vasculature, which have been reported to synthesize VEGF.[29–31] Immunolocalization studies using cell-specific markers are underway and will identify the cell type(s) involved in VEGF expression.

The physical and metabolic changes in the retina following laser vein occlusion may be complex, but a predominant change is local hypoxia. Pournaras et al[32,33] used O_2-sensitive micro-electrodes to characterize oxygen levels in a related model of branch vein occlusion in the miniature pig. They found that the inner retina becomes hypoxic following branch retinal vein occlusion, with the lowest pO_2 measurements at a 50% retinal depth, corresponding to the interface of the inner nuclear layer and the outer plexiform layer. Our findings of increased VEGF mRNA in this layer are consistent with a hypoxia-induced upregulation of VEGF following laser vein occlusion.

Pan-retinal photocoagulation is used clinically to treat iris and retinal neovascularization that occurs secondary to diabetic retinopathy and retinal vein occlusion. In this treatment, large areas of retina are ablated using laser photocoagulation, and distant neovascularization on the optic nerve or iris subsequently regresses. Possible explanations for the efficacy of this treatment include: destruction of cells producing an angiogenic factor, attenuation of the metabolic demand, induction of an angiogenic inhibitor, and reduction of hypoxia by increasing diffusion from the choroid. Pournaras et al measured pO_2 in the pig retina after retinal vein occlusion followed by pan-retinal photocoagulation and found that the procedure reduced hypoxia in the retina.[33] Our findings suggest that neovascularization associated with retinal vein occlusion results from a hypoxia-induced upregulation and release of VEGF; thus pan-retinal photocoagulation, by reducing hypoxia, may down-regulate VEGF leading to regression of neovascularization.

Many ophthalmic diseases, including proliferative diabetic retinopathy, are characterized by ischemia and neovascularization, and VEGF may play an important role in mediating these diseases. Preliminary results measuring vitreous levels of VEGF in eight diabetics and 12 control patients undergoing vitrectomy, indicate that VEGF is significantly elevated in the vitreous of patients with proliferative diabetic retinopathy.[34] If further investigations corroborate the role of VEGF in mediating neovascular eye diseases, then therapies directed at blocking the production or action of VEGF may have significant clinical application. Recent studies in tumor models using VEGF neutralizing antibodies suggest that such an approach is feasible.[35]

There is increasing evidence that VEGF and its receptors serve an important role in various angiogenesis-dependent processes. Millauer et al have demonstrated increased expression of flk-1, a high-affinity receptor for VEGF, in endothelial cells and perivascular tissue in embryonic mice compared to adult tissue.[36] High levels of VEGF mRNA have been demonstrated in the rat corpus luteum compared to the mural granulosa cells at a time corresponding to maximal angiogenesis, suggesting a physiological role for VEGF in ovulation.[30] Bovine aortic smooth muscle cells express VEGF mRNA and secrete a VEGF-like endothelial cell mitogen, and binding sites for VEGF have been demonstrated in quiescent vessels *in vivo*,[30] implying that VEGF may have a role in maintaining normal vasculature. A particularly high density of binding sites for VEGF have been found on adult rat heart valves and aorta.[30,37] In vascular regions subjected to high or turbulent flow, there may be a need for a factor to maintain the endothelial integrity.

Weindel et al found that cells cultured from acquired immune deficiency syndrome-related Kaposi's sarcoma, a vascular cell tumor, expressed more VEGF mRNA than normal human umbilical vein endothelial cells or human vascular smooth muscle cells.[29] High levels of VEGF mRNA have been demonstrated in various tumors including, human adenocarcinoma and glioblastoma, a highly vascularized tumor, in which VEGF mRNA expression was particularly high in tissue adjacent to necrotic centers, presumed to be hypoxic.[12,14,15] Thus VEGF seems to play an important role in development, in physiological maintenance of vessels, in physiological ovulation, and in pathological tumor growth. Our findings suggest an important role for VEGF in pathological ocular neovascularization, which further supports its identity as a universal angiogenic factor.

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References

1. Ashton N: Retinal vascularization in health and disease. Am J Ophthalmol 1957, 44:7–24
2. Michaelson IC: The mode of development of the vascular system of the retina, with some observations on its significance for certain retinal disease. Trans Ophthalmol Soc UK 1948, 68:137–180
3. D'Amore PA, Glaser BM, Bruson SK, Fenselau AH: Angiogenic activity from bovine retina: partial purification and characterization. Proc Natl Acad Sci USA 1981, 78:3068–3072
4. Sivalingam A, Kenney J, Brown GC, Benson WE, Donoso L: Basic fibroblast growth factor levels in the vitreous of patients with proliferative diabetic retinopathy. Arch Ophthalmol 1990, 108:869–872
5. Senger DR, Van De Water L, Brown LF, Nagy JA, Yeo K-T, Yeo T-K, Berse B, Jackman RW, Dvorak AM, Dvorak HF: Vascular permeability factor (VPF, VEGF) in tumor biology. Cancer Met Rev 1993, 12:303–324
6. Dvorak HF, Orenstein NS, Carvalho AC, Churchill WH, Dvorak AM, Galli SJ, Feder J, Bitzer AM, Rypysc J, Giovinco P: Induction of a fibrin-gel investment: an early event in line 10 hepatocarcinoma growth mediated by tumor-secreted products. J Immunol 1979, 122:166–174
7. Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT: Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science 1989, 246:1309–1312
8. Leung DW, Cachianes G, Kunang W-J, Goeddel DV, Ferrara N: Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 1989, 246:1306–1309
9. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak JF: Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 1983, 219:983–985
10. Connolly DT, Heuvelman DM, Nelson R, Olander JV, Eppley BL, Delfino JJ, Siegel NR, Leimgruber RM, Feder J: Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. J Clin Invest 1989, 84:1470–1478
11. Shima DT, Adamis AP, Yeo K-T, Yeo T-K, Berse B, Brown L: Hypoxic regulation of vascular permeability factor (vascular endothelial growth factor) mRNA and protein secretion by human retinal pigment epithelial cells. Invest Ophthalmol Vis Sci (suppl) 1993, 34:990
12. Shweiki D, Itin A, Soffer D, Keshet E: Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 1992, 359:843–845
13. Dvorak HF, Sioussat TM, Brown LF, Verse B, Nagy JA, Sotrel A, Manseau EJ, Van De Water L, Senger DR: Distribution of vascular permeability factor (vascular endothelial growth factor) in tumors: concentration in tumor blood vessels. J Exp Med 1991, 174:1275–1278
14. Brown LF, Berse B, Jackman RW, Tognazzi K, Manseau EJ, Senger DR, Dvorak HF: Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in adenocarcinomas of the gastrointestinal tract. Cancer Res 1993, 53: 4727–4735
15. Plate KH, Breier G, Weich HA, Risau W: Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. Nature 1992, 359:845–848
16. Pournaras CJ, Tsacopoulos M, Strommer K, Gilodi N, Leuenberger PM: Experimental retinal branch vein occlusion in miniature pigs induces local tissue hypoxia and vasoproliferative microangiopathy. Ophthalmology 1990, 97:1321–1328
17. Miller JW, Stinson WG, Folkman J: Regression of experimental iris neovascularization with systemic alpha-interferon. Ophthalmology 1993, 100:9–14
18. Virdi PS, Hayreh SS: Ocular neovascularization with retinal vascular occlusion. I. Association with experimental retinal vein occlusion. Arch Ophthalmol 1982, 100:331–341
19. D'Anna SA, Hochheimer BF, Joondeph HC, Graebner KE: Fluorescein angiography of the heavily pigmented iris and new dyes for iris angiography. Arch Ophthalmol 1983, 101:289–293
20. Yeo K-T, Sioussat TM, Faix JD, Senger DR, Yeo T-K: Development of time-resolved immunofluorometric assay of vascular permeability factor. Clin Chem 1992, 38:71–75
21. Yeo K-T, Wang HH, Nagy JA, Sioussat TM, Ledbetter SR, Hoogewerf AJ, Zhou Y, Masse EM, Senger DR, Dvorak HF, Yeo T-K: Vascular permeability factor (vascular endothelial growth factor) in guinea pig and human tumor and inflammatory effusions. Cancer Res 1993, 53:2912–2918
22. Sioussat TM, Dvorak HF, Brock TA, Senger DR: Inhibition of vascular permeability factor (vascular endothelial growth factor) with antipeptide antibodies. Arch Biochem Biophys 1993, 301:15–20
23. Stata Corporation: State Reference Manual: Release 3.1. Edited by College Station, Texas, 1993
24. Snedecor GW, Cochran WG: Statistical Methods. Iowa State University Press, Ames, 1980
25. Friedman M: The use of ranks to avoid the assumption of normality implicit in the analysis of variance. J Am Stat Assoc 1937, 32:675–701
26. Sambrook J, Fritsch EF, Maniatis T: In Molecular Cloning. A Laboratory Manual. Edited by Cold Spring Harbor Press, New York, 1989
27. French-Constant C, Van De Water L, Dvorak HF, Hynes RO: Reappearance of an embryonic pattern of fibronectin splicing during wound healing in the adult rat. J Cell Biol 1989, 109:903–914

28. Berse B, Brown LF, Van De Water L, Dvorak HF, Senger DR: Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors. Mol Biol Cell 1992, 3:211–220
29. Weindel K, Marme D, Weich HA: AIDS-associated Kaposi's sarcoma cells in culture express vascular endothelial growth factor. Biochem Biophys Res Commun 1992, 183:1167–1174
30. Ferrara N, Houck KA, Jakeman LB, Winer J, Leung DW: The vascular endothelial growth factor family of polypeptides. J Cell Biochem 1991, 47:211–218
31. Plouet J, Chollet P, Moro F, Malecaze F: Secretion ofVAS/VEGF by retinal pericytes: a paracrine stimulation of endothelial proliferation. Invest Ophthal Vis Sci 1993, 34:900
32. Pournaras CJ, Tsacopoulos M, Riva CE, Roth A: Diffusion of O2 in normal and ischemic retinas of anesthetized miniature pigs in normoxia and hperoxia. Graefe's Arch Clin Exp Ophthalmol 1990, 228:138–142
33. Pournaras CJ, Tsacopoulos M, Strommer K, Gilodi N, Leuenberger PM: Scatter photocoagulation restores tissue hypoxia in experimental vasoproliferative microangiopathy in miniature pigs. Ophthalmology 1990, 97:1329–1333
34. Adamis AP, Miller JW, Bernal MT, D'Amico DJ, Folkman J, Yeo T-K, Yeo K-T: Elevated vascular permeability factor/vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. Am J Ophthalmol (in press)
35. Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrara N: Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. Nature 1993, 362:841–844
36. Millauer B, Wizigmann-Voos S, Schnurch H, Martinez R, Moller NPH, Risau W, Ullrich A: High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. Cell 1993, 72:835–846
37. Jakeman LB, Winer J, Bennett GL, Altar A, Ferrara N: Binding sites for vascular endothelial growth factor are localized on endothelial cells in adult rat tissues. J Clin Invest 1992, 89:244–253